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Review

Characteristics of advanced methods used for typing bacterial isolates from mastitis with particular reference to Staphylococci

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Abstract

Staphylococci are very common human and animal pathogens. A variety of staphylococcal virulence determinates leads to vast range of infections. One of them is mastitis which is a common disease of the mammary glands. The incidence of this disease is widespread all over the world and depends on bacterial virulence and on prevention programs. The influence of mastitis on human health is not globally evaluated, however, in veterinary fields loses in milk production caused by bovine mastitis are a constant economic problem. One of the most important parts of the mastitis control programs is accurate diagnosis of the inflammation and characterization of the etiological factors which leads to reduction of mastitis spread. Recent reports show that staphylococci are common bacterial etiological factors of mastitis, and this paper is an overview of the diagnostic typing methods used for characterization of staphylococcal isolates. A number of different techniques available to applicate is described. Phenotypic methods to identify and to differentiate isolates or discriminate virulence factors are still in use, however, some advanced genetic methods offering higher discriminatory power are reported as more accurate. In fact, nowadays the most powerful tool on that field is next generation sequencing (NGS) of the whole genome, but its high cost and requirement of special laboratory equipment makes it hard to use for routine diagnostics. That is why standard PCR techniques-based methods, and the sequencing of particular genes, are mostly used for typing bacterial isolates. Most of these techniques are characterized by a high discriminatory power, big epidemiological concordance, and repeatable results. The presented report describes the techniques used most frequent in mastitis diagnostics related to staphylococci typing and shows their advantages and disadvantages.

Key words: coagulase-negative staphylococci, genetic methods, mastitis, phenotypic methods, *Staphylococcus aureus*

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Introduction

Staphylococci exist in most of natural and industrial niches. As commensals, the bacteria occur mostly in skin, in skin glands, and in mucous membranes of healthy human and animal individuals. As opportunistic pathogens under particular conditions staphylococci cause infections of various course, localization or manifestation. To date, the bacteria were assigned to in over 50 staphylococcal species including two groups: coagulase-positive (CoPS), and coagulase-negative staphylococci (CoNS). The division into those two groups is based on difference in ability of plasma clotting of cells. The coagulase-positive staphylococci belong to *Staphylococcus aureus and S. intermedius* group (SIG group), and they are recognized as bacteria more virulent than CoNS. Otherwise, the CoNS comprise a big reservoir of genes, and some of them originate from CoPS. Moreover, there are evidences of CoNS infections, especially in patients with immune system suppression (Savini et al. 2016). In bovine mastitis, the CoNS are the second the most frequent group of etiological factors after streptococci (Malinowski et al. 2003). A variety of staphylococcal virulence determinants leads to vast range of infections. *S. aureus* is well known as responsible for skin and soft tissues infections, endocarditis, meningitides, respiratory tracts, nephritis, osteomyelitis, toxic shock syndrome, blood infections with sepsis, or foodborne diseases (Plata et al. 2009). Staphylococci are common pathogens of mammary glands (Gelasakis et al. 2015). CoNS are traditionally regarded as minor mastitis pathogens in comparison to *S. aureus* but recent data show the increasing significance of CoNS as mastitis-causing agent (Pitkala et al. 2004, Pyorala and Taponen 2009).

Mastitis is generally defined as inflammation of mammary glands (MG), commonly caused by bacterial infections (Brenaut et al. 2014). This disease occurs mostly in animals but reports show, that it also emerges in acute phase in approximately 20% of breastfeeding women who experience a red, painful breast with an accompanying fever (Begovic et al. 2013, Cullinane et al. 2015). Traditionally recognized mastitis presents subclinical, latent or clinical manifestations. In the first stage, the presence of infection is observed, but the only evidences of infection are increased number of somatic cells and pathogen presence. The asymptomatic course of the disease often results in a persistent infection for a long period of time, known as the latent infection. In this stage, the pathogens are isolated from milk, however neither manifestation nor increased number of somatic cells are reported. Clinical mastitis is the stage when the symptoms of inflammatory response are clearly visible. An increase in inflammation reveals local signs such as abnormality in milk properties or changes in mammary gland tissue (De Vliegher et al. 2012, Thompson-Crispi et al. 2014).

Mastitis is a disease, which strongly depends on environmental factors, such farming conditions that facilitate the development of mastitis. First of all, various concentrations of somatic cells in milk are a good indicator of a cow's health, and not only indicate inflammatory response of the mammary gland but they also affect the quality of milk. Milk with a cell count of up to 400 000/ml is considered fit for consumption, while the number of somatic cells threshold value of a healthy udder is up to 200 000/ml (Janus and Borkowska 2008, Pilarska 2014). It is very important also to recognize the economic impact of mastitis. It is one of the most frequent diseases in dairy animals, it is caused by many pathogens and it generates very high economical looses (Haugaard et al. 2012). The economical problems mostly harm farmers who are engaged in milk or meat production. Today, the value of global milk production is estimated as EUR 260 billion/year. According to the NMC (National Mastitis Council 1996), 65% of costs caused by mastitis on a farm is related to the decrease in the productivity of cows. The second reason of looses is lack of profits (23%) including differences between cows' health state and cost regarding regeneration of production capacity. Indirect costs, however, include future reproductive problems. The remaining cost components include treatment costs and veterinary services (5%), waste milk (6%) from the infected quarter and extra labor associated with handling a sick animal (1%). That is the reason why fast and reliable technique for staphylococci determination is needed. For veterinarians and diagnosticians as well, it is important to collect the milk samples using a proper techniques. Only correctly collected samples can give reliable microbiological results and the standards are presented in the Codex Alimentarius (FIL-IDF 1981).

Due to the high rate of infections and the increasing resistance to a variety of antibiotics, it is crucial to find a diagnostic method which will allow for accurate typing of the analyzed pathogen. In a clinical setting, the most common are phenotypic methods. Standard biochemical tests and antibiotic susceptibility testing play a major role in the routine diagnostics. However, genetic methods in bacterial typing present a higher discriminatory power and epidemiological concordance (Capurro et al. 2009). This is the reason why fast diagnostic methods based on molecular techniques are strongly needed. In laboratory practice, the key question is to find a "gold standard" for identifying a pathogen which plays a main role in mastitis (Sabat et al. 2013). This paper presents an overview of the

most frequently used methods for typing *Staphylococcus* spp., applied also in mammary gland infections diagnostics.

Phenotypic methods

Phenotypic methods are routinely used in microbiological laboratories. These tests represent the first step in identifying mastitis-related agents in most cases. Commercial biochemical tests show a lower discriminatory power than genetic methods. Capurro reports than only 61% of mastitis pathogens were correctly identified using biochemical tests (Capurro et al. 2009). Progress in genetics has allowed for a more specific identification of bacteria at a species level, as well as to detect genes responsible for biochemical factors like enzymes. However, biochemical tests are still needed to confirm activity of the gene in phenotype. Moreover, antibiotic resistance testing gives one of the most important clinical parts of information about the pathogen (Bengtsson et al. 2009).

Antibiotic resistance testing

A high number of veterinary and medical data reported indicates the importance to introduce complex therapeutic protocols that will provide fast information on the changes in pathogen resistance. The most valuable factor regarding bacterial resistance is introduction of proper targeted therapy (Bengtsson et al. 2009). The methods used for antibiotic susceptibility testing are the disc-diffusion plate test by Kirby-Bauer, the agar screen test, the E-test, or MIC (Minimal Inhibitory Concentration- a method based on the microdilution of an antibiotic), (Parisi et al. 2016). The first method is based on the diffusion of the antibiotic from the disc into a medium. An antibiotic's specific concentration inhibits bacterial growth in the zone around the disc. In the agar screen test, the main role is played by agar plates containing the appropriate concentration of an antibiotic. An E-test shows inhibition of bacterial strain growth around the strip saturated by a defined gradient of an antibiotic. In laboratory practice, tests usually include at least six to up to twenty-one antibiotics (Begovic et al. 2013, Parisi et al. 2016). The most popular antimicrobial agents used in susceptibility testing are: azithromycin, chloramphenicol, ciprofloxacin, clindamycin, doxycycline, penicillin, erythromycin, gentamicin, sulfamethoxazole/trimethoprim, tetracycline, vancomycin, and cefoxitin or oxacillin (Jagielski et al. 2014, Pu et al. 2014, Li et al. 2015).

An emerging concern is the increasing presence of MRSA (Methicillin Resistant *Staphylococcus aureus*) and VRSA (Vancomycin Resistant *Staphylococcus aureus*) strains. This phenomenon is usually related with the multidrug resistance of isolates (Nemeghaire et al. 2014, Budd et al. 2015, Wang et al. 2015, Fernandes dos Santos et al. 2016). Choosing of the right antimicrobial indicator to estimate methicillin resistance is very important. Some authors propose oxacillin and cefoxitin, but there are differences in detecting methicillin resistance between them (Parisi et al. 2016). Furthermore, strains carrying the *mec*A gene but are susceptible to oxacillin were determined falsely resistant (false MSSA phenotype). This suggests to use not only biochemical tests but also to supplement testing with genetic identification (Pu et al. 2014).

An increase of multidrug resistance in mastitis-associated staphylococci was observed. In 2015 in China, a high percentage of macrolides, lincosamides and streptogramines (MLS) resistance phenotype were described (Li et al. 2015). An opposite situation to that observed in China was found in a study conducted in Greece where at the same time (2015) almost all of the strains were multidrug susceptible (Zdragas et al. 2015). However, data shows that resistance to antibiotics occurs in a majority of MRSA strains, but in MSSA strains, resistance to antibiotics other than methicillin was reported (Silva et al. 2013). Beside the *S. aureus* strains, an increase of resistance in other coagulase-positive and coagulase-negative staphylococci was also shown (Sampimon et al. 2011, Fernandes dos Santos et al. 2016, Taponen et al. 2016). Some papers report that in subclinical mastitis in animals mostly bovine or ovine pathogens are usually susceptible to many antibiotics and they are rarely resistant to more than one antibiotic. The occurrence of MRSA strains is also low. However, some studies show that livestock animals represent a great reservoir of many mastitis-associated agents (Bengtsson et al. 2009). Even nasal strains, which do not cause any clinical manifestation, may demonstrate a multidrug resistance profile (Nemeghaire et al. 2014). In conclusion, antibiotic resistance in mastitis-associated agents is variable, mostly depending on therapy practice (Parisi et al. 2016). Li et al. report that the occurrence rate of resistance genes is higher than that of phenotypic resistance, which suggests using appropriate antibiotics (Li et al. 2015). Hence, it is crucial to monitor trends in antimicrobial resistance in mastitis-associated agents (Jagielski et al. 2014, Wang et al. 2015).

Commercial biochemical tests

Commercial biochemical tests are used as the first step to mastitis-associated strain identification at

Table 1. The most frequent tests used to measure the enzymatic activity of mastitis-associated isolates.

Test	Enzyme	Function	References
Haemolysis	Haemolysins	membrane-damaging toxins; role in invasion and persistence;	(Cremonesi et al. 2015)
Coagulase production	Coagulase	plasma clotting; strain identification and grouping;	(Monecke et al. 2011, Rabello et al. 2007)
Catalase production	Catalase	catalytic H_2O_2 decomposition; strain identification and grouping;	(Rabello et al. 2007)
Proteolysis	Proteases	catalytic proteins decomposition; role in invasion and persistence;	(Miedzobrodzki et al. 2002, Sabat et al. 2008)
Lipolysis	Lipase	catalytic lipid decomposition; role in invasion;	(Begovic et al. 2013)
Nuclease production	Nuclease	catalytic DNA decomposition; role in persistence;	(Zastempowska et al. 2014)
Fibrynolysis	Fibrynolysin	catalytic fibrin decomposition; role in invasion;	(Aarestrup et al. 1995)

a species level. The API 20 Staph system (bioMerieux), the API ID 32 Staph (bioMerieux), (Capurro et al. 2009), the Staph-Zym (Rosco), (Capurro et al. 2009), the Vitek system (bioMérieux), (Schlotter et al. 2014), Microgen Staph ID (Microgen® Bioproducts) (Zdragas et al. 2015), the STAPHYtest16 (Pliva-Lachema), and additionally some other non-commercial tests, composed of specific combinations of enzymes based on biochemical reactions are used in diagnostic laboratories (Zadoks and Watts 2009).

However, these commercial biochemical tests also demonstrate some limitations. The most important issue is the high variability of strains which means that isolates belonging to the same species can show different enzyme expression. In addition, phenotypic identification is much more subjective than genotypic tests. These arguments negatively affect the typeability of commercial biochemical tests (Zadoks and Watts 2009).

MALDI-TOF method

The MALDI-TOF (Matrix Assisted Laser Desorption Ionization – Time Of Flight) system allows identification at a species level with high sensitivity (Taponen et al. 2016). Frey et al. report that 97,8% of the analyzed strains were correctly identified using this system (Frey et al. 2013). This method is based on measuring the mass spectrum of peptides, which is species-specific. The role of correct and specific identification depends on the increasing number of inflammation cases caused by CoNS. Coagulase-negative staphylococci belong to a variable group, which constitute a good reservoir for antibiotic resistance genes and causes infections of mammary glands. For that reason, typing of mastitis-associated CoNS strains is an important goal for diagnostics (El-Ashker et al. 2015, Taponen et al. 2016). The MALDI-TOF system represents a very useful alternative method to the genetic identification of mastitis pathogens (Krol et al. 2016).

Enzymatic activity

Enzyme production is one of the bacterial strategies that ensure survival in the host organism. The staphylococcal enzymes belong to a big group of virulence factors and knowledge of the peptide secretion profiles is very important in monitoring the infections. Table 1 shows the most frequent tests used to recognize particular enzymes in mastitis.

Genetic methods

To achieve results with a high discriminatory power and good epidemiological concordance, it is necessary to use methods based on genetic engineering techniques (Zadoks and Watts 2009). The PCR-based techniques are a great tool for confirming gene presence or specifying an allelic variant. Methods consisting of restriction analysis show differences between strains with a very high level

Table 2. An overview of the genes detected by PCR amplification in mastitis-associated isolates. The most frequent genes are in bold.

Function/Factor	Genes	Function	References
Resistance genes	*fos*B	Putative marker for fosfomycin, bleomycin	(Puacz et al. 2015)
	blaZ, *bla*I, *bla*R	Penicillin resistance	(Sampimon et al. 2011, Xu et al. 2015)
	***SSCmec, mec*A**	Methicillin resistance	(Aras et al. 2012)
	*tet*K, *tet*L,	Tetracycline resistance	(Taponen et al. 2016)
	RPP	Ribosomal protection proteins	(Begovic et al. 2013)
	erm A-C	Methylase genes	(Puacz et al. 2015)
	van A-C	Vankomycin resistance	(Xu et al. 2015)
	*mph***C**	Phosphotransferase genes	(Li et al. 2015)
	*ere*A	Erythromycin resistance	(Li et al. 2015)
	vga A,C,E	Streptogramin A resistance	(Wendlandt et al. 2015)
	msr A, B	Macrolide efflux determinants	(Xu et al. 2015)
	lnu A	Lincosamide resistance	(Xu et al. 2015)
	lsa A,B,C,D	Pleuromutilin-lincosamide-streptogramin A resistance	(Wendlandt et al. 2015)
	*sal*A	Pleuromutilin resistance	(Wendlandt et al. 2015)
	nuc	Thermonuclease	(Shuiep et al. 2009)
	agr	Accessory gene regulation	(Xu et al. 2015)
Enterotoxins	*ent* A-R	Enterotoxins A-R	(Puacz et al. 2015)
	sea-e, g-o	Enterotoxins a-e, g-o	(Shuiep et al. 2009)
Haemolysins	*luk*F, R, E, B, D, M,	Leukocidins	(Cosandey et al. 2016)
	hla, *hlb*, *hld*	Haemolysins	(Ote et al. 2011, Xu et al. 2015)
Proteases	*Aur*	Aureolysin	(Sabat et al. 2008)
	*ssp*A, *ssp*B, *ssp*P	Serine proteases	(Ote et al. 2011)
	*spl*A, *spl*B, *spl*E	Serine proteases	(Ikawaty et al. 2010)
	*et*A, *et*B, *edt*	Exfoliative toxins	(Aires-de-Sousa et al. 2007)
Other exotoxins	*Tst*	Toxic shock syndrome toxin-1	(Ote et al. 2011, Xu et al. 2015)
Adhesin and biofilm formation proteins	***ica*A, *ica*C, *ica*D**	Intracellular adhesion proteins	(Xu et al. 2015)
	coa	Coagulase	(Shuiep et al. 2009, Aras et al. 2012)
	*clf*A, *clf*B,	Clumping factor	(Cosandey et al. 2016)
	fib	Fibrinogen-binding protein	(Puacz et al. 2015)
	*fnb*A, *fnb*B	Fibronectin-binding proteins	(Xu et al. 2015)
	map	MHC class II analog protein	(Xu et al. 2015)
	vwb	Willebrand Factor – binding protein	(Puacz et al. 2015)
	*atl*E	Autolysin/Adhesin AtLE	(Begovic et al. 2013)
	can	Collagen-binding protein	(Xu et al. 2015)
	*ebp*S	Cell surface elastin-binding protein	(Salaberry et al. 2015)
	*sdr*C, *bbp*	Bone sialoprotein-binding protein	(Ote et al. 2011; Xu et al. 2015)
	eno	Laminin binding protein	(Ote et al. 2011)
	*ebp*S	Elastin binding protein	(Salaberry et al. 2015)
	bap	Biofilm associated protein	(Salaberry et al. 2015)
Capsule	*capH*5	CP5 synthesis enzyme	(Xu et al. 2015)
	*capH*8	CP8 synthesis enzyme	(Xu et al. 2015)

cont. Table 2

Function/Factor	Genes	Function	References
Immune evasion proteins	***spa***	Protein A (IgG binding protein)	(Hata 2016)
	scn	Staphylokinase	(Hata 2016)
	sak	Staphylococcal complement inhibitor (SCIN)	(Puacz et al. 2015)
	chp	Chemotaxis inhibitory protein (CHIPS)	(Wendlandt et al. 2015, Xu et al. 2015)
	lg	IG binging protein	(Xu et al. 2015)
Other	*RS*	16S – 23S rRNA	(Cosandey et al. 2016)
	*aro*A	EPSP synthase[1]	(Saei et al. 2009)

[1] 5-enolpyruvylshikimate-3-phosphate synthase. Catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) to produce enolpyruvyl shikimate-3-phosphate and inorganic phosphate. UniProtKB – Q05615 (AROA_STAA8) (http://www.uniprot.org/)

of sensitivity. Nowadays, the most reliable results are generated by methods that offer sequencing of genes. In this paper, the most frequent methods are described.

Polymerase Chain Reaction (PCR) amplification

The polymerase chain reaction (PCR) method and its variants represent the most frequently used genetic technique in laboratory practice. Depending on the aim of a particular study, it is possible to choose a specific and sensitive test. A variety of PCR methods have been described, confirming the presence of a targeted gene. For example, the real-time variant of PCR that gives a quantitative assessment of the product. Additional techniques such restriction digestion improve standard PCR methods offering special supplementary results as discrimination of individual isolates. Even small quantities of DNA or RNA (RT-PCR- reverse transcriptase PCR) can be detected (Klein et al. 2012). These methods are commonly used in diagnosing mastitis, mostly for estimating the relatedness between staphylococci or detecting an important gene, like a virulence factor. Table 2 shows an overview of the genes which can be detected by PCR amplification.

Pulse-field gel electrophoresis (PFGE) typing

Pulse-field gel electrophoresis (PFGE) is one of the most frequent methods used for the genetic analysis of isolates from mastitis (Peton and Le Loir 2014, Lundberg et al. 2016). For the last decade, PFGE has been recommended as a primary typing tool for staphylococci responsible for infections of mammary glands. The method is applied in large scale epidemiological research by analyzing centre-to-centre transmission events (Fijalkowski et al. 2013, Sabat et al. 2013, Hata 2016).

PFGE is based on cleavage of purified DNA sample using a restriction enzyme that recognizes infrequently occurring restriction sites in the genome of a pathogen. The next step includes separating cleaved genome fragments by pulse-field electrophoresis in agarose gel. This type of electrophoresis consists of changes in the direction of the electric field (Sabat et al. 2013). In most cases, as the restriction endonuclease the *Sma*I enzyme is used. But there are reports using the *Apa*I or *Sst*I for restriction analysis, most commonly as supplementary tests (Fessler et al. 2010, Bardiau et al. 2013).

The big advantage of PFGE is the ability to analyze long fragments of DNA, from 30kb to over 1Mb. This method is reliable, relatively cheap, it has a high discriminatory power and great epidemiological concordance, so in laboratory practice it proves the high effectiveness of results (Dingwell et al. 2006). Owing to intra-laboratory programs of methodology optimization PFGE is becoming a standardized analytical tool used to distinguish differences among isolates. Moreover, it was estimated that plasmid DNA does not interfere with the macrorestriction pattern, which gives more reliable comparisons (Sabat et al. 2013). A major disadvantage, however, is that this method requires extensive labor, is technically demanding and time-consuming (Dingwell et al. 2006). Other limitations of this method include: analysis is prone to subjectivity, several problems regarding the portability of results to other laboratories and continuous quality control, in comparison with sequence-based methods (Sabat et al. 2013).

However, it is still one of the most powerful tools used to analyze clonal relationships (Rabello et al. 2007, Zdragas et al. 2015). Investigators described PFGE as the most discriminant genetic method. For

that reason, PFGE is used as a "gold standard" and in many evaluations of other genetic tests based on comparison with this method and their discriminatory power (Mitra et al. 2013, Jagielski et al. 2014). That is why it is a very good candidate method to be used in veterinary microbiology to examine staphylococci group other than just *S. aureus*, such as CoNS, atypical strains and strains from dynamic infections that have particularly arisen in man, cows or other animals (Begovic et al. 2013).

Multilocus Sequence Typing (MLST)

Alternatively to PFGE, the multilocus sequence typing (MLST) technique is used to distinguish polymorphic patterns in bacterial populations and differences between the isolates. According to databases, it is also the second most frequent used method in the examination of staphylococci responsible for mastitis (Jagielski et al. 2014, Cremonesi et al. 2015, Mazzilli et al. 2015, Rota et al. 2015). Because it is capable of detecting important genes, the MLST method plays a main role in the epidemiological investigation of mastitis cases (Peton and Le Loir 2014).

The MLST method was inspired by the phenotypic MLEE (Multilocus Enzyme Electrophoresis) technique. Multilocus sequence typing involves PCR amplification of seven housekeeping genes and their sequencing (Wang et al. 2015). Target loci depend on the pathogen species and in *Staphylococcus aureus* cases the *arc*, *aro*E, *glp*F, *gmk*, *pta*, *tpi* and *yqi*L genes are compared (Parisi et al. 2016). Differences in allelic variants and their combination have an arbitrarily assigned number (Zastempowska et al. 2014). On this basis, the sequence types are detected (Sabat et al. 2013). A useful tool is the online international database (Based Upon Related Sequence Types, eBURST), which allows to determine the genetic relatedness between the analyzed mastitis-associated isolates. This analysis leads to the creation of MLST-maps and helps monitor the propagation of individual strains around the world (Sabat et al. 2013).

The MLST technique is specific and unequivocal, which constitutes its greatest advantage. It also has a standardized nomenclature, it is highly reproducible and allows to compare results in an online database (Rabello et al. 2007, Delgado et al. 2011). As the PFGE method, MLST is also time-consuming and labor-extensive, but PFGE generates a higher discriminatory power. An important disadvantage of MLST is its high costs of sample analysis, in comparison with other genetic methods (Sabat et al. 2013). However, it is a great technique to distinguish the variability of strains and specification of epidemiological changes among staphylococcal strains causing inflammation of mammary glands.

Single locus sequence typing (SLST) typing

To determine the relationships and differences between mastitis-associated isolates it is possible to use single locus sequence typing (SLST). The method is based on PCR amplification and sequencing of the single target gene. There are some variants of this method depending on discrimination of the particular analyzed gene. In mastitis cases, most SLST analyses concentrate on *S. aureus,* but it can be used for other staphylococci as well. This method of typing can also be used to determine bacterial species, like 16S rRNA gene typing. In the present paper, the most popular SLST variants are described.

Staphylococcal protein A gene (*spa*) typing

Typing of the *Staphylococcus aureus* protein A gene (*spa*) is one of the most commonly used techniques based on single locus sequencing (Sabat et al. 2013). It is a very popular method used for genotyping staphylococci from mastitis (Budd et al. 2015, Kahila Bar-Gal et al. 2015, Lundberg et al. 2016, Parisi et al. 2016). In databases, most papers used *spa* gene typing as a main method or a supplementary tool.

Spa typing is based on polymorphism and variable number of 24bp repeats detected after sequencing the previously amplified *spa* gene. This gene encodes the staphylococcal protein A which takes part in bacterial cell binding to the Fc fragment of the type G immunoglobulin. This binding results in loss of IgG activity, and indirectly affects the complement (Mitra et al. 2013, Sabat et al. 2013).

Although *spa* typing demonstrates a lower discriminatory power than pulse-field gel electrophoresis, this technique is characterized by several advantages. The SLST method for *spa* gene is described as cost-effective, especially in comparison with MLST, and as a fast and easy technique for standard use in laboratory. Moreover, *spa* typing has excellent reproducibility and even more importantly – high stability, and a standardized intra-laboratory nomenclature. These features make it a great tool for international cooperation. Additional advantages are high-throughput due to the StaphType software, and full portability of data via the Ridom database, which confirms utility and reliability in worldwide studies (Sabat et al. 2013). For comprehensive results, it was investigated that using *spa* typing in combination with other

methods like PFGE, is also a good strategy (Mitra et al. 2013).

Staphylococcal chromosome cassette *mec* (SSC*mec*) typing

Typing of SCC*mec* demonstrates very important epidemiological characteristics. The increasing number of MRSA isolates in mastitis-associated pathogens has led to a modification of the basal method using PCR amplification to detect the SSC*mec* cassette and add sequencing of amplified product. As well as PCR-based methods, this supplementary sequencing technique is very useful in analyzing mastitis isolates and in general diagnostics (Fessler et al. 2010, Frey et al. 2013, Pu et al. 2014, Fernandes dos Santos et al. 2016).

Typing of the staphylococcal chromosome cassette *mec* (SCC*mec*) elements allows to categorize the methicillin-resistant *S. aureus* (MRSA) as well as methicillin-resistant coagulase-negative staphylococci (MRCNS). The *mec*A gene is harbored by chromosomal cassette encoding penicillin-binding protein 2a *(PBP2a)* (Becker et al. 2014), determining resistance to methicillin and additionally to a majority of beta-lactam antibiotics. Presence of the SCC*mec* cassette in the cell genome describes it as a multidrug-resistant pathogen. For that reason, the significant role of SCC*mec* typing is estimated.

Comparative genomic hybridization: microarrays

Microarray probes are used in mastitis laboratory practice in two ways. The first technique was designed for detecting bacterial strains in milk samples. It is a rapid and effective test for confirming inflammation caused by staphylococci. Species identification by microarray tests is based on immobilized oligonucleotide probes which are unique for an individual pathogen. After adding the milk sample, when the pathogen is present, it binds to the oligonucleotide stabilized by linear polymer containing photo-reactive groups. Then, the DNA found in the milk sample is amplified by PCR and hybridized to a microarray plate. As a result, the species of pathogen is determined (Green et al. 2015).

The second application of the microarray method is the characterization of strain genes, and on this basis, assigning them to proper clonal complex. Comparative genomic hybridization uses microarrays based on hybridization of pure bacterial DNA to labeled specific fragments of nucleic acids (about 200 bp) or oligonucleotides (about 70 bp) on the microarray plate. This binding is ensured by chemical groups or an enzymatic reaction. Then, unbound DNA is removed by washing, and the properly hybridized nucleic acids are measured automatically by a properly equipped scanner. This technique allows to detect many different pathogen genes with high sensitivity (Sabat et al. 2013, Kahila Bar-Gal et al. 2015). There are a few commercial microarray tests available. For *S. aureus* the Alere Technologies GmbH, Jena, Germany, test was designed that analyses 170 genes and their allelic variants simultaneously. It is one of the most frequently used methods of genotyping pathogens infecting mammary glands (Schlotter et al. 2014, El-Ashker et al. 2015, Puacz et al. 2015). Summing up, it allows to analyze 334 target sequences at the same time (Monecke et al. 2011). Microarrays allow to detect several important genes, including genes specific for *S. aureus*, and many virulence factors like accessory gene regulator (*agr*) alleles, toxins, enterotoxins, putative toxins, haemolysins, proteases and biofilm formation molecules as well as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), capsule type-specific genes, and a variety of antibiotic resistance genes (Green et al. 2015).

Microarray comparative genomic hybridization is a very specific and sensitive method which allows for fast and cost-efficient analysis of whole pure genomic probes. It is a technique with high epidemiological concordance and gives reliable data to compare bacterial isolates with each other and distributes them to specific clonal complexes. However, the biggest disadvantage is its possibility to only detect known alleles of genes, so it is useless for detecting new single nucleotide mutations. Despite this, it is still one of the best tests for mastitis-related agents (Sabat et al. 2013).

Next generation sequencing (NGS)

It seems that NGS is the most reliable and effective method which is used nowadays. There is an increasing tendency to use whole genome sequencing in the study of infections of mammary glands. Because of the high costs of analysis, it is still less popular than other genetic methods even though the number of cases when NGS is used rises every year (Bardiau et al. 2016). However, the costs of whole genome sequencing are continuing to decline, so in the near future there is a high possibility it will become a very powerful tool in the routine identification of mastitis related organisms (Sabat et al. 2013). The biggest difference between NGS and traditional Sanger sequencing is a relatively short time of analysis, because of the ability to generate millions of reads of short

sequences. That indicates a highly effective detection of sequences of the whole genome of the pathogen (Sabat et al. 2013).

Conclusions

Identification of mastitis-associated bacteria is made possible by a variety of methods. For staphylococci, the majority of techniques are designed for *S. aureus*. Although there is an increasing number of tests that have the ability to detect and describe the phenotype and genotype of other species, including coagulase-negative staphylococci (CoNS). Recent papers state that the biggest challenge for investigating the pathogen infecting the mammary gland is to monitor epidemiological strains or gene transmission around the world and study why opportunistic species becomes pathogenic. Progress in genetic engineering offers more modifications to known methods or leads to designing new, advanced techniques which lead to the better analysis of pathogens.

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